

Application No.: 09/973,088  
Amendment Dated 19 October 2005  
Reply to Office Action of 19 May 2005

*AMENDMENTS TO THE SPECIFICATION*

Please amend paragraph [0073] to read as follows.

[0073] Plants could not be regenerated from transgenic pine lines that were treated using a stringent washing process involving multiple lengthy washes to obtain complete eradication of the *Agrobacterium*, using nylon membranes for cell collection. Plants have subsequently been regenerated from transgenic pine lines recovered using this improved eradication process, with fewer washes of shorter duration facilitated by use of the supports in the method disclosed here following *Agrobacterium* transformation. ~~Presence of the transgenes~~

Please amend paragraph [0099] to read as follows.

[0099] Loblolly and hybrid pine cell lines were used which had been grown and maintained as described in Examples 1-2 above. In order to test selection improvements that would be carried out alone or in combination with eradication procedures following *Agrobacterium* transformation, without confounding any growth effect related to the *Agrobacterium* gene transfer process and unrelated to the selection and eradication methods *per se*, transformed lines resistant to GENETICIN® were generated by the bombardment method described in U.S. patent application Serial No. 09/318,136 filed on 25 May 1999, now U.S. Patent No. 6,518,485, and New Zealand Patent No. 336149, each incorporated herein by reference.

Please amend paragraph [0100] to read as follows.

[0100] Specifically, to prepare for gene transfer, a sterile fabric support (here NITEX, commercially available from Sefar Inc.) was placed in a sterile Buchner funnel and one to five milliliters of

Application No.: 09/973,088

Amendment Dated 19 October 2005

Reply to Office Action of 19 May 2005

embryogenic suspension was pipetted onto the fabric support such that the embryogenic tissue was evenly distributed over the surface. The liquid medium was suctioned from the tissues using a mild vacuum. The fabric support with embryogenic tissue was removed from the Buchner funnel and placed on a GELRITE solidified DCR<sub>3</sub> preparation medium (Table 2) in 100 X 25 mm plastic petri dishes. Dishes were incubated in a dark growth chamber at 23°C ± 2°C for about 24-48 hours. The preparation medium of U.S. Patent No. 6,518,485 contains 30 g maltose and 70 g PEG.

Please amend paragraph [0116] to read as follows.

[0116] In this example, transformation, selection, and eradication experiments were conducted using somatic embryogenic cell lines from five different *Pinus radiata* families wherein a standard commonly-used somatic embryogenesis process was followed and, by making only the changes taught in the method described in this application in the preceding examples, transgenic *Pinus radiata* was produced. In the above examples, the media described in cited U.S. Patents as being sufficient to promote growth and embryogenesis of southern yellow pines and hybrids were adapted by our method to create media for the purposes of eradicating *Agrobacterium* and selecting transformants. In the present example, the maintenance media described in U.S. Patent 5,565,355 (which is hereby incorporated by reference) as being sufficient to promote growth of *P. radiata* are adapted by our improved method to create preparation, recovery, selection, and eradication media for the purposes of transforming *P. radiata* somatic embryogenic cells with *Agrobacterium*, eradicating *Agrobacterium* and selecting transformants. These examples serve to illustrate that any nutrient media that have been established as sufficient to promote growth or embryogenesis of the target tissue may be employed in conjunction with the present method without undue experimentation. The maintenance medium of U.S. Patent No. 5,565,355 is:

Application No.: 09/973,088  
Amendment Dated 19 October 2005  
Reply to Office Action of 19 May 2005

### Standard Embryogenesis Medium (embryogenic tissue maintenance medium):

<u>Major ion stock</u>	<u>40 ml</u>
<u>Minor ion stock</u>	<u>20 ml</u>
<u>Iron chelate stock</u>	<u>20 ml</u>
<u>Vitamin stock</u>	<u>10 ml</u>
<u>Inositol</u>	<u>1.0 gm</u>
<u>Sucrose</u>	<u>30.0 gm</u>
<u>Difco Bacto agar</u>	<u>8.0 gm</u>

(Adjust pH to 5.6-5.8 before addition of agar and autoclaving. Add filter sterilized amino acids after autoclaving.)

### Major Ion Stock (make up to 400 ml):

<u>Compound</u>	<u>Weight (gm)</u>
<u><math>\text{KNO}_3</math></u>	<u>14.31</u>
<u><math>\text{MgSO}_4 \cdot 7\text{H}_2\text{O}</math></u>	<u>4.00</u>
<u><math>\text{CaCl}_2 \cdot 2\text{H}_2\text{O}</math></u>	<u>0.25</u>
<u><math>\text{NaNO}_3</math></u>	<u>3.10</u>
<u><math>\text{NH}_4\text{H}_2\text{PO}_4</math></u>	<u>2.25</u>

Minor Ion Stock (make up to 200 ml):

<u>Compound</u>	<u>Weight (mg)</u>
<u>MnSO<sub>4</sub>•4H<sub>2</sub>O</u>	<u>36.0</u>
<u>H<sub>3</sub>BO<sub>3</sub></u>	<u>80.0</u>
<u>ZnSO<sub>4</sub>•7H<sub>2</sub>O</u>	<u>250.0</u>
<u>KI</u>	<u>10.0</u>
<u>CuSO<sub>4</sub>•5H<sub>2</sub>O</u>	<u>24.0</u>
<u>Na<sub>2</sub>MoO<sub>4</sub>•2H<sub>2</sub>O</u>	<u>2.0</u>
<u>CoCl<sub>2</sub>•6H<sub>2</sub>O</u>	<u>2.0</u>

Iron Stock (make up to 1 liter):

<u>FeSO<sub>4</sub>•7H<sub>2</sub>O</u>	1.5 gm
<u>Na<sub>2</sub>EDTA</u>	2.0 gm

## Amino Acids:

amino acid amount

Application No.: 09/973,088  
Amendment Dated 19 October 2005  
Reply to Office Action of 19 May 2005

<u>glutamine</u>	<u>110 mg/L</u>
<u>asparagine</u>	<u>105 mg/L</u>
<u>arginine</u>	<u>35 mg/L</u>
<u>minor amino acids stock</u>	<u>2 ml/L</u>

Minor Amino Acids Stock (make up to 800 ml):

<u>Amino Acid</u>	<u>weight (gm)</u>
<u>citrulline</u>	<u>1.58</u>
<u>ornithine</u>	<u>1.52</u>
<u>lysine</u>	<u>1.10</u>
<u>alanine</u>	<u>0.8</u>
<u>proline</u>	<u>0.7</u>

(Dispense into 40 ml aliquots. Freeze immediately, store frozen, and thaw only on day of use. Adjust pH to 5.6-5.8 and filter sterilize before use.)

Please amend paragraph [0119] to read as follows.

[0119] As can be seen in Table 14, for no cell line was the average growth over a period of six weeks less for cells grown over support membranes and biphasic treatments than for cells grown directly on gelled medium. Because the membrane supports facilitate rapid transfer and weighing with minimal manipulation of the cells, damage that cells sustain during transfer between gelled media without membrane supports, as described in Example 2 above, may account for some of the difference between Treatment A and the other treatments. Also, for all *P. radiata* cell lines, as had been shown for *P. taeda* and *P. rigida* hybrids in Example 2 above, growth on a polyester membrane support was superior to growth on a nylon membrane support or a filter paper support alone. The same patterns were observed in data analyzed for a single two-week transfer period. Embryos were subsequently successfully developed, matured, and germinated from *P. radiata* cells of these lines that had been maintained on polyester support membranes. These data suggested that polyester membrane supports could be used to facilitate washing, eradication and selection following

Application No.: 09/973,088

Amendment Dated 19 October 2005

Reply to Office Action of 19 May 2005

*Agrobacterium* transformation of *P. radiata* as they had been used for *P. taeda* and *P. rigida* hybrids in the methods described in Examples 3, ~~5, and 6~~ 4 and 5 above. This also illustrates that the present invention is not limited to any single basal culture nutrient medium formulation. It should be understood that any nutrient media commonly used in *Pinus* somatic embryogenesis will be suitable for use with this method.